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(71) Applicants *(for all designated States except US)*: VLAAMS INTERUNIVERSITAIR INSTITUUT VOOR BIOTECHNOLOGIE (VIB) [BE/BE]; Center for Transgene Technology and Gene Therapy (CTG), Herestraat 49, Campus Gasthuisberg O & N, B-3000 Leuven (BE). LEUVEN RESEARCH & DEVELOPMENT VZW (LRD VZW) [BE/BE]; Groot Begijnhof, Benedenstraat 60 bus 3, B-3000 Leuven (BE).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: CARMELIET, Peter [BE/BE]; Sapellenbos 10, B-3052 Oud-Heverlee (BE). COLLEN, Désiré [BE/GB]; 28 Collingham Gardens, London SW5 0HN (GB). ANGELILLO-SCHERRER, Anne [CH/CH]; 50, rue de la Gare, CH-1163 Etoy (CH).

(74) Agents: BIRD, William, E. et al.; Bird Goën & Co, Vilvoordsebaan 92, B-3020 Winksele (BE).

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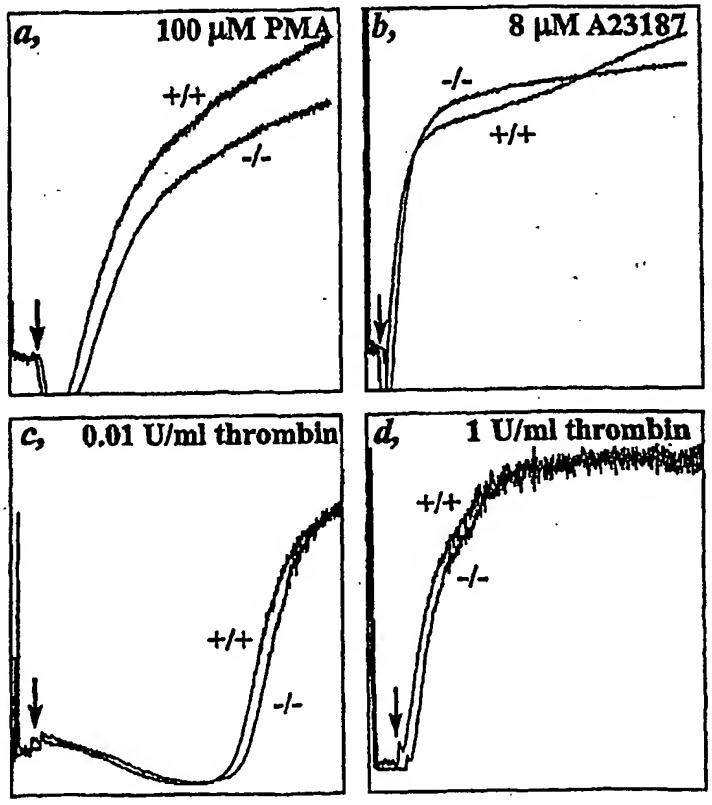


(57) Abstract: Inhibition of a growth arrest-specific gene (Gas6) function or of a Gas6 receptor is used for the prevention or treatment of a thromboembolic disease or a thrombotic pathologic condition in a mammal. The invention further provides a pharmaceutical composition comprising an inhibitor of a growth arrest-specific gene (Gas6) function or of a Gas6 receptor as an active ingredient in admixture with at least a pharmaceutically acceptable carrier.

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# USE OF INHIBITION OF A Gas6 FUNCTION OR OF A Gas6 RECEPTOR FOR PREVENTING AND TREATING A CARDIOVASCULAR DISEASE.

The present invention relates to a new method for the prevention and treatment of a thromboembolic disease such as arterial or venous thrombosis, based on the inhibition of, e.g. based on the administration of an inhibitor of, a growth arrest-specific gene 6 (Gas6) function or of a Gas6 receptor.

## BACKGROUND OF THE INVENTION

The major factors involved in the patho-physiology of thrombosis are abnormalities of the vessel wall, alterations of blood flow, and changes in the composition of the blood. Arterial and venous thrombosis and their complications, which include focal ischemic cerebral infarction (ischemic stroke), acute myocardial infarction and venous thromboembolism among others, represent the major cause of morbidity and death in the developed countries of the world.

Platelets play a central role in arterial thrombosis. They adhere to exposed subendothelial matrix proteins and become activated. They change their shape and then aggregate. Tissue factor (TF) is thought to be the primary initiator of *in vivo* blood coagulation. In the absence of TF expression, endothelial cells actively maintain thromboresistance. Vascular wall damage exposes TF which binds activated factor VII (factor VIIa). The factor VIIa-TF complex then triggers thrombin generation by activating factors IX and X. In addition to activating platelets, thrombin converts fibrinogen to fibrin, amplifies its own generation by activating factors V and VIII, and then activates factor XIII which finally stabilizes the fibrin clot, according to Bates et al. in *Cardiovasc. Res.* (1999) 41:418-432 and Davie E.W. in *Thromb. Haemost.* (1995) 74:1-6. Prevention and treatment of thrombosis are therefore based on the administration of either antiplatelet drugs or anticoagulants, or of a combination of both.

One of the inherited risk factors for thrombosis is protein S deficiency. Protein S, a vitamin K-dependent plasma protein, serves as a cofactor for the anticoagulant activity of an other vitamin K-dependent protein, activated protein C (APC). The protein C anticoagulant system provides important control of the

blood coagulation cascade by degrading coagulation factors Va and VIIIa according to B. Dahlback in *Thromb.Haemost.* (1991) 66:49-61. Resistance to APC is the most common form of inherited thrombosis disease according to B.Dahlback in *Blood* (1995) 85:607-614.

In order to investigate the mechanism controlling growth arrest in mammalian cells, a set of six growth arrest-specific (hereinafter "Gas") genes have been cloned and sequenced. Gas6 was originally identified as a gene whose expression in mouse fibroblasts increased during serum starvation and was described in detail, together with its human homolog, by Manfioletti et al. in *Mol. Cell Biol.* (1993) 13(8):4976-4985 and U.S.Patent No. 5,538,861.

The protein encoded by Gas6 is a vitamin K-dependent protein related to protein S (i.e. human Gas6 cDNAs encode a protein having 44% amino acid sequence identity to human protein S) which is suspected to play a role in a number of biological processes, namely the regulation of a protease cascade relevant in cell growth regulation, according to Matsubara et al. in *Dev.Biol.* (1996) 180:499-510. Both molecules comprise a gamma-carboxyglutamic acid rich region (i.e. the A domain), four epidermal growth factor (EGF)-like repeats (forming the C domain) and a carboxyterminal tandem globular (G) region with homology to the steroid hormone binding globulin (SHBG) protein (i.e. the D domain). However, in contrast to protein S, Gas6 lacks a loop which is crucial for the anticoagulant activity of protein S, according to Manfioletti et al. (cited *supra*).

The Axl receptor, disclosed by O'Bryan et al. in *Mol. Cell Biol.* (1991) 11: 5016-5031, was identified due to its ability to render mouse fibroblast cells tumorigenic. Axl expression appears to have profound effects on the growth state of cells. U.S.Patent No. 5,538,861 discloses that Gas6 is a ligand for the Axl receptor. The cDNA sequence of the receptor tyrosine kinase Rse, that is preferentially expressed in the adult brain, was described by Mark et al. in *J.Biol. Chem.* (1994) 269:10720. cDNA sequences encoding proteins identical to human and human Rse have been termed Sky and Tyro3 respectively and disclosed by Ohashi et al. in *Oncogene* (1994) 9:699 and by Lai et al. in *Oncogene* (1994) 9:2567 respectively. Rse is structurally related to Axl (also known as Ufo or Ark) and shares 43% overall amino acid sequence identity

with this tyrosine kinase receptor. Rse and Axl, together with c-Mer (also known as Eyk or Nyk) disclosed by Graham et al. in *Cell Growth Differ.* (1994) 5:647, define a class of tyrosine kinase receptors whose extracellular domains resemble neural cell recognition and adhesion molecules. Like Rse, Axl is also expressed in the nervous system, but is more widely expressed than Rse in peripheral tissues. Gas6 binds members of the above-mentioned class of tyrosine kinase receptors according to Nagata et al. in *J. Biol. Chem.* (1996) 271(47):30022-30027 and Crosier et al. in *Pathology* (1997) 29(2):131-135.

The extracellular domains of these receptors comprise two immunoglobulin (Ig)-like repeats followed by two fibronectin type III repeats, found in cell adhesion molecules. The Axl receptor is capable of homophilic binding as well as binding to Gas6. However, Axl is not only expressed as a transmembrane protein, but is also cleaved in the extracellular domain to generate a soluble Axl form, which has been detected in conditioned media of Axl expressing cells, serum, plasma, brain, liver, spleen and tumor cells. Soluble Axl could act as a competitive inhibitor for Gas6 by sequestering free Gas6 or could bind to Axl transmembrane receptor. Binding of soluble Axl to Axl transmembrane receptor might give a signal distinct from Gas6 or inactivate Axl transmembrane receptor on the cell surface according to Costa et al. in *J. Cell Physiol.* (1996) 168(3):737-744 and Varnum et al. in *Nature* (1995) 373:623-626.

Gas6 and Axl are expressed by vascular endothelial cells according to Varnum et al. (cited *supra*). Gas6 has been reported to inhibit homophilic Axl-mediated aggregation of myeloid cells according to Avanzi et al. in *Blood* (1998) 91(7):2334-2340, but cell-bound Gas6 may mediate aggregation of myeloid cells via interaction with Axl receptor on adjacent cells according to McCloskey et al. in *J. Biol. Chem.* (1997) 272(37):23285-23291. Gas6 does not affect adhesion of granulocytes to resting endothelial cells, while it inhibits granulocyte adhesion to TNF-α activated endothelial cells at high concentrations according to Avanzi et al. (cited *supra*). Gas6 is mitogenic for fibroblasts according to Goruppi et al. in *Oncogene* (1996) 12(3):471-480 and for Schwann cells according to Li et al. in *J. Neurosci.* (1996) 16(6):2012-9 and

U.S.Patent No. 5,714,385, but not for myeloid cells according to Avanzi et al. in *Exp. Hematol.* (1997) 25(12):1219-1226 or endothelial cells. Gas6, induced in injured vascular smooth muscle cells, induces Axl-mediated chemotaxis of smooth muscle cells and, although not mitogenic by itself, enhances the mitogenic activity of thrombin according to Fridell et al. in *J. Biol. Chem.* (1998) 273(12):7123-7126. Gas6 also acts as a survival factor for serum-starved fibroblasts and GnRH neuronal cells, presumably via activation of PI3-kinase and Akt kinase according to Goruppi et al. in *Mol. Cell Biol.* (1997) 17(8):4442-4453. Axl signaling protects against apoptosis as Axl deficient fibroblasts cannot be rescued by Gas6 after serum-withdrawal according to Bellosta et al. in *Oncogene* (1997) 15(20):2387-2397.

## SUMMARY OF THE INVENTION

In a first aspect, the present invention relates to the use of inhibition of a growth arrest-specific gene (Gas6) function or of a Gas6 receptor (for instance by means of an inhibitor or antagonist such as a Gas6 function neutralizing antibody, or by means of a ribozyme or an antisense RNA directed against Gas 6 or a Gas 6 receptor function) for the manufacture of a medicine for the prevention or treatment of a cardiovascular disease other than resulting from an endothelial dysfunction, e.g. a disease caused by platelet aggregation, in particular a thromboembolic disease or a thrombotic pathologic condition in a mammal, preferably in a human. Within the framework of this invention, the growth arrest-specific gene (Gas6) receptor to be inhibited preferably is a tyrosine kinase receptor such as the Axl receptor, the Rse receptor, the c-Mer receptor or fragments thereof. Inhibition of the Gas6 function may also be effected by means of a protease able to cleave the extracellular domain of the Axl receptor, preferably within the sequence VKEPSTPAFSWPWW.

Inhibition according to this invention also includes inhibition of the native protein or polypeptide encoded by Gas6 or of a modified form thereof, for instance a form including a modified gamma-carboxyglutamic acid rich region (i.e. the A domain) - such as disclosed in U.S.Patent No. 6,017,882 – that enhances membrane binding affinity of the protein relative to the corresponding native protein.

Examples of thromboembolic diseases or thrombotic pathologic conditions within the scope of this invention namely include:

- ischemic diseases such as ischemic stroke or ischemic cerebral infarction, acute myocardial infarction, chronic ischemic heart disease.
- an ischemic disease of an organ other than myocardium or a region of the brain, for instance a peripheral limb.
- venous thromboembolism.
- arterial or venous thrombosis.
- pulmonary embolism.
- restenosis following coronary artery bypass surgery or following percutaneous transluminal angioplasty of coronary artery.
- disseminated intra-vascular coagulation.

As far as prevention is concerned, non-limiting examples of thrombotic pathologic conditions within the scope of this invention namely include, in addition to the above:

- the relapse of coronary thrombosis after acute myocardial infarction.
- coronary thrombosis in patients with unstable angina pectoris.
- cerebral ischemic infarction (ischemic stroke) in patients with atrial fibrillation.
- arterial thrombosis after vascular surgery.
- the occlusion of arterio-venous shunt in dialysis patients.

In a second aspect, the present invention relates to a pharmaceutical composition comprising an inhibitor or antagonist of a Gas6 function or of a Gas6 receptor, or a ribozyme or an antisense RNA directed against Gas 6 or a Gas 6 receptor function, or a protease able to cleave the extracellular domain of the Axl receptor as a first active ingredient in admixture with at least a pharmaceutically acceptable carrier, the said pharmaceutical composition being

preferably intended for the prevention or treatment of a cardiovascular disease other than resulting from an endothelial dysfunction, e.g. a disease caused by platelet aggregation, in particular a thromboembolic disease or a thrombotic pathologic condition, such as above defined. The said pharmaceutical composition may further optionally comprise a thrombolytic agent, preferably in respective proportions with the said first active ingredient such as to provide a synergistic effect in the said prevention or treatment.

In another aspect, the present invention relates to the use of inhibition, for instance by means of an inhibitor or antagonist, of a growth arrest-specific gene (Gas6) function or of a Gas6 receptor during extracorporeal blood circulation and hemodialysis, i.e. in a method for treating blood from a mammal, in order to prevent platelet activation leading to thrombus formation in the extracorporeal system and – because of excessive platelet – bleeding in the patient. In yet another aspect, the present invention relates to the use of inhibition, for instance by means of an inhibitor or antagonist, of a growth arrest-specific gene (Gas6) function or of a Gas6 receptor as a diagnostic tool or agent, for instance in order to identify, via protein or mRNA or DNA characterization, individuals having a predisposition to acquire a a thromboembolic disease or a thrombotic pathologic condition, such as above defined.

Finally, the present invention provides a method of prevention or treatment of a cardiovascular disease other than resulting from an endothelial dysfunction, e.g. a disease caused by platelet aggregation, in particular a thromboembolic disease or a thrombotic pathologic condition (such as above defined) in a mammal, preferably a human, comprising administering to a mammal in need of such prevention or treatment a therapeutically effective amount, i.e. preferably an amount able to protect the patient against thromboembolism without causing bleeding side effects, of an inhibitor of a Gas6 function or of a Gas6 receptor, or a ribozyme or an antisense RNA directed against Gas 6 or a Gas 6 receptor function, or a protease able to cleave the extracellular domain of the Axl receptor.

## BRIEF DESCRIPTION OF THE DRAWINGS